EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Institute for Reference Materials and Measurements

The certification of reference plasmas for the prothrombin time BCR-628 (Abnormal Plasma 2) BCR-630 (Normal Plasma) BCR-631 (Abnormal Plasma 1)

T.W. Barrowcliffe, A.R. Hubbard, S. Margetts, L.J. Weller, J. MacNab, D. Bennink, B.M. Gawlik, C.L. Klein, A. Lamberty

Report EUR 21060

The mission of IRMM is to promote a common European measurement system in support of EU policies, especially health and consumer protection, environment, agriculture, internal market and industrial standards.

European Commission
Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements

Contact information
European Commission
Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements
Retieseweg 111
B-2440 Geel • Belgium

Tel.: +32 (0)14 571211
Fax: +32 (0)14 590406

http://www.irmm.jrc.be
http://www.jrc.cec.eu.int

EUR Report 21060
Luxembourg: Office for Official Publications of the European Communities

ISBN 92-894-5172-6

Printed in Belgium

European Commission

BCR information
REFERENCE MATERIALS

The certification of reference plasmas for the prothrombin time

T.W. Barrowcliffe, A.R. Hubbard, S. Margetts, J. Weller
J. MacNab

National Institute for Biological Standards and Control
Hertfordshire EN6 3QG - United Kingdom

D. Bennink

European Commission DG RTD
Standards, Measurements and Testing Programme, B-1049 Brussels - Belgium

B.M. Gawlik, C.L. Klein, A. Lamberty

European Commission DG Joint Research Centre
Institute for Reference Materials and Measurements - B-2440 Geel - Belgium

Report EUR 21060 EN

ABSTRACT

The aim of the present project was to provide an alternative method of estimating INR (International Normalised Ratio) which will lead to increased inter-laboratory agreement and also overcome the need for time-consuming local calibration exercises which are currently required to compensate for differences in instrumentation and reagents. It is proposed to use three freeze-dried pooled plasmas (one normal plasma pool and two abnormal plasma pools with increased INR values) each with an assigned INR value to construct a calibration curve by plotting local prothrombin time (PT) vs. assigned INR value. The INR of test plasmas can then be interpolated from local PT estimates.

Three definitive preparations consisting of a pooled normal plasma and two pools of patient plasma (1 medium and 1 high INR range) were prepared for INR calibration in a collaborative study involving 20 laboratories. Estimates of homogeneity (by weight and PT estimation) and stability indicated that the preparations were suitable for use as reference preparations. INR values were estimated using three International Reference Thromboplastin preparations (BCT/253 - human, RBT/90 - rabbit, OBT/79 - bovine). Inter-laboratory variability of INR estimates was lowest with the normal plasma (maximum GCV 4.32%) and highest with the high INR patient plasma (maximum GCV 10.99%). The low variability was the main reason for significant differences between some mean INR estimates with different thromboplastin reagents. The largest difference was seen with abnormal plasma-2 between the INR estimate with OBT/79 (3.20) and the estimates with RBT/90 (3.93) and BCT/253 (3.75) and this invalidated the assignment of a mean INR value calculated from the results obtained with all three thromboplastin reagents. The assigned INR values are the geometric means of the INR estimates obtained by the individual laboratories using BCT/253 and RBT/90. These assigned values are intended to be used with all thromboplastin reagents of human or rabbit origin. The assigned INR values are as follows:

BCR No	*Material*	*INR value*[1)]	*Uncertainty*[2)]	*No of accepted results*
630	Normal plasma	0.99	0.02	40
631	Abnormal plasma-1	2.38	0.06	40
628	Abnormal plasma-2	3.84	0.12	40

[1)] These values are the geometric means from 40 independent estimates

[2)] These values are the half width of the 95% confidence intervals of the mean INR values

LIST OF ABBREVIATIONS AND DEFINITIONS

APTT activated partial thromboplastin time - a screening test sensitive to defects associated with the intrinsic pathway of blood coagulation (1)

BCT/253 WHO International Biological Reference Preparation for Thromboplastin (human brain derived)

CV coefficient of variation

FV coagulation factor V

FVII coagulation factor VII

GCV geometric coefficient of variation - defined as (GSD - 1) x 100 % where GSD is the geometric standard deviation (2)

HEPES N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]

HIV human immune deficiency virus

INR international normalised ratio - a measurement based on the prothrombin time of patients' plasma indicating the intensity of oral anticoagulant therapy (3)

ISI international sensitivity index - a measure of the responsiveness of a thromboplastin reagent to the degree of anticoagulation in plasma induced by oral anticoagulants (3)

IU International Unit - the concentration of blood coagulation factors (eg Factors VIII, II, VII, IX, X) in International Standards established by the World Health Organisation. The IU is defined by the relevant WHO International Standard for that parameter (4)

MNPT mean normal prothrombin time - the geometric mean of prothrombin times from at least 20 fresh plasma samples from healthy individuals (3)

NEQAS national external quality assurance scheme - a national scheme operated in the United Kingdom to monitor the performance of clinical laboratories in the measurement of parameters in the field of blood coagulation

OBT/79 WHO International Biological Reference Preparation for Thromboplastin (human brain derived)

PL NIBSC Thromboplastin reagent 3 used for this project

PT prothrombin time - a screening test sensitive to defects associated with the extrinsic pathway of blood coagulation (1)

RBT/90 WHO International Biological Reference Preparation for Thromboplastin (rabbit brain derived)

X/95 NIBSC Thromboplastin reagent 1 used for this project

Y/95 NIBSC Thromboplastin reagent 2 used for this project

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Background: need for certified reference materials

The prothrombin time (PT) test is the most frequently performed coagulation test and is routinely carried out to monitor the effectiveness of oral anticoagulation therapy. Consistency of therapeutic approaches between different centres makes it vital that test results from different laboratories are comparable. Currently the PT value of the patient's plasma is reported as a ratio to the PT value found with normal plasma and adjusted to an International Normalised Ratio (INR) using a correction factor (called the International Sensitivity Index or ISI) in order to compensate for variability caused by the use of different thromboplastin reagents. The INR value is adimensional. Despite the use of the "*INR system*" there is evidence from the national NEQAS surveys that considerable inter-laboratory variability still exists in INR determination. The remaining variability appears to be caused by the use of different instrumentation and different sources of normal plasma. Ideally each laboratory should adopt a local system ISI, which is suited to the instrumentation and thromboplastin reagent, used and also establish a local mean normal prothrombin time (MNPT) by taking the geometric mean PT from 20 normal individuals. This process, which should be repeated with each new batch of thromboplastin reagent, is very time-consuming and hence many laboratories accept manufacturers' assigned ISI values and also use various commercial normal reference plasmas.

The objective of the present exercise was to prepare stable freeze-dried reference plasmas, with assigned INR values, which can be used to standardise the determination of INR values in patient plasmas without the need to determine a local MNPT or a local ISI. The aim is to calibrate three freeze-dried plasmas - one normal plasma and two abnormal plasmas with increased INR values. These calibrated plasmas could then be used to construct a calibration curve of assigned INR vs. local prothrombin time which would allow the interpolation of the prothrombin time of any test plasma to give an INR value (Figure 1.1).

1.2 Choice of the material for the BCR preparations

Routine measurement of PT for monitoring oral anticoagulant therapy is carried out on freshly collected citrated plasma samples. The ideal reference materials should therefore also consist of fresh citrated plasma. However, practical issues such as stability, storage and transport make the use of fresh and also frozen plasma impossible. Preliminary studies which evaluated freeze-dried plasmas in sealed glass ampoules indicated that freeze-dried plasmas prepared from pooled normal plasma and patient plasma would be best suited to the intended use (see Section 3).

1.3 Design of the project

Preliminary studies on trial fills (small-scale) of freeze-dried pooled plasmas were used to evaluate the optimal conditions for preparation of the materials and also for comparing abnormal plasmas prepared artificially or obtained from patients (see section 3). Three plasma pools (one normal and two abnormal plasma pools) were ampouled in 1995 as the candidate reference materials. Homogeneity and accelerated degradation studies together with the certification exercise were completed in 1996.

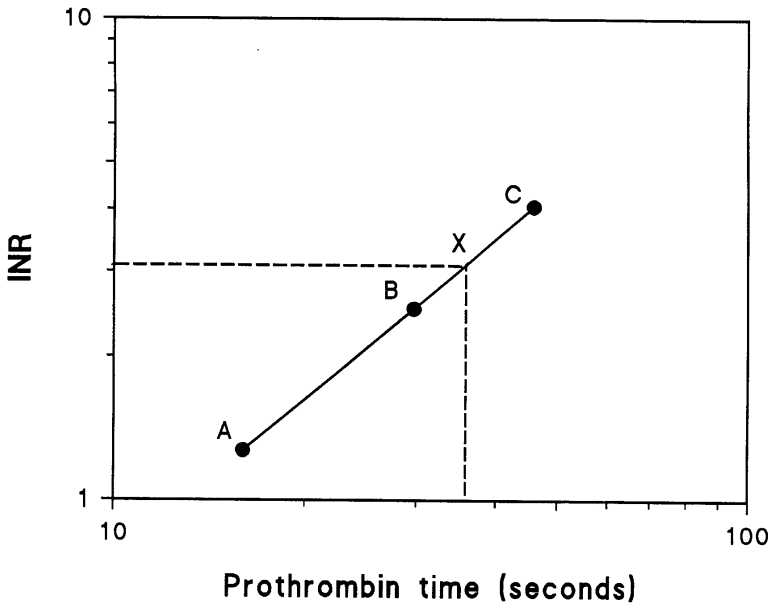

Figure 1.1 - Illustration of the use of calibrated plasmas (A, B, C) for the interpolation of the value of test plasma "X" from the local prothrombin time.

2. PARTICIPANTS

Preparation and ampouling of materials

- Division of Haematology and Division of Standards Processing
  National Institute for Biological Standards and Control, Potters Bar UK

Homogeneity and stability testing

- Division of Haematology and Division of Standards Processing
  National Institute for Biological Standards and Control, Potters Bar UK

Certification Measurements (20 laboratories)

- A Bianchi Bonomi Istituto Medicina Interna, Milan IT
  and Control, Potters Bar UK
- Departamento de Hematologia, Fundacion Favaloro, Buenos Aires AR
- Dept. of Clinical Chemistry & Transfusion Medicine
- Dept. of Clinical Chemistry, Ribe County Hospital, Esbjerg, DK
- Dept. of Haematology, RELAC, University Hospital Leiden
  NL
- Dept. of Haematology, Royal Hallamshire Hospital, Sheffield UK
- Dept. of Haematology, Royal Postgraduate Medical School
- Dept. of Transfusion Medicine, University of Freiburg DE
- Division of Haematology National Institute for Biological Standards
- Divisione Medica, Centro Emostasi, Ospedali Riuniti, Parma IT
- Gradipore Ltd., North Ryde NSW AU
  Hammersmith Hospital, London UK
- Lab Central d'Hematologie, Hotel Dieu, Paris FR
- Medical Clinic, Haematology Laboratory, Ullevaal Hospital, Oslo NO
- MRC Epidemiology & Medical Care Unit, St Bartholomew's Hospital, London UK
- Research Centre, Hamilton Civic Hospitals, Hamilton CA
  Sahlgren's University Hospital, Goteborg SE
- Seccion de Hemostasia, Hospital "Princeps d'Espana", Barcelona ES
- Servei d'Hematologia, Hospital de la Santa Creu y Sant Pau, Barcelona ES
- Servico de Patologia Clinica, Hospital de Santa Cruz, Carnaxide PT
- Servizio di Angiologia e Malattie della Coagulazione, Ospedale S Orsola, Bologna IT
- Thrombosis Reference Centre, Withington Hospital, Manchester UK

2.5 Statistical Analysis

- Informatics Laboratory
  National Institute for Biological Standards and Control, Potters Bar UK

3. PRELIMINARY STUDIES

3.1 Effect of buffering and freeze-drying on the prothrombin time

Initial studies focussed on the conditions for buffering which would produce a freeze-dried plasma with a PT value very similar to that found with fresh, unbuffered, plasma (typical of clinical samples). Freeze-drying unbuffered plasma produced an increase in the PT value. This increase could be reduced by the addition of HEPES buffer to the plasma and a final concentration of 20 mmol/l was chosen in order to achieve the PT (31.9 seconds) closest to fresh, unbuffered plasma (31.7 seconds) (Table 3.1).

Table 3.1 - Effect of buffering and freeze-drying on PT of pooled abnormal plasma

Sample	*Buffering (HEPES mmol/l)*	*Prothrombin time (s)*	
		Fresh	Freeze-dried
1	No buffer	31.7	43.6
2	10	36.1	34.5
3	20	37.0	31.9
4	40	40.6	34.4

PT values are means of at least 6 estimates using Manchester reagent (rabbit brain thromboplastin).

3.2 Evaluation of trial fill plasma preparations

An evaluation exercise was organised in early 1995 to compare the inter-laboratory variability of INR estimation using either calibrated reference plasmas or conventional INR estimation and also to compare the use of freeze-dried patient and artificially depleted plasmas. Five small trial plasma fills were freeze-dried for the evaluation exercise - one pooled normal plasma, two pooled abnormal patient plasmas (1 medium and 1 high INR) and two abnormal plasmas (1 medium and 1 high INR) prepared artificially using aluminium hydroxide adsorption. Six laboratories were asked to determine the INR of these plasmas by conventional means (relative to the MNPT) using their normal reagents and instruments, which are listed in Table 3.2.

The combined mean INR for each preparation was then assigned to the plasmas and two calibration curves were then constructed using either the normal plasma and the two abnormal patient plasmas or the normal plasma and the two artificial plasmas by plotting assigned INR against the local prothrombin time. The INR of a test freeze-dried patient plasma (F) was then determined by interpolation from the local prothrombin time on the two calibration curves. The INR of plasma F was also determined in each laboratory by the conventional method relative to the MNPT. The results in Table 3.3 indicate that the combined mean INR for sample F was very similar by the two methods, however, the inter-laboratory variability of the INR values obtained by interpolation was approximately half that obtained by the conventional method. Furthermore the use of patient or artificial plasmas for the interpolation exercise were associated with similar inter-laboratory variability. These results indicated that the use of calibrated plasmas for INR interpolation would lead to improved inter-laboratory agreement. The results from the evaluation of the trial fills have been published (5).

Table 3.2 - Reagents and Instruments used by individual laboratories for evaluation of trial fills

Lab	*Thromboplastin*	*ISI*	*MNPT (s)*	*Coagulometer*
1	Manchester 'traditional'	1.12	13.1	KC4
2	Neoplastin	1.84	12.6	STA
3	Thromboplastin C	2.17	9.51	ACL 300
	Thromborel S	1.08	12.62	S & G
4	IL PT HS Plus	1.20	13.6	ACL 300
5	Thromboplastin IS	1.09	14.7	S & G
6	Recombiplastin	0.88	9.6	ACL 300

Table 3.3 - INR values for sample F

INR Method	*Geometric Mean*	*Range*	*GCV%*
Conventional (vs MNPT)	2.73	2.41 - 3.08	8.92
Interpolation (combined data)			
vs. normal + patient plasmas	2.70	2.55 - 2.85	3.44
vs. normal + artificial plasmas	2.70	2.55 - 2.90	4.92

3.3 Patient plasma vs. artificially depleted plasma

It was intended that the definitive reference plasmas would be calibrated for INR using the primary International Reference Thromboplastins (RBT/90-rabbit, BCT/253-human, OBT/79-bovine) and that preferably only a single combined mean INR value would be assigned to each reference plasma. In order to check the feasibility of assigning a single INR value to the definitive fills two laboratories estimated the INR of the trial fill plasmas using the International Reference Thromboplastins. The results in Table 3.4 indicate that similar INR values are obtained with all three thromboplastin reagents for the normal and patient plasmas indicating that a single combined INR may be feasible. However, INR estimations of the two artificial plasmas were not the same with all three reagents since RBT/90 was associated with increased INR values compared to BCT/253 and OBT/79. Based on these results it was decided to prepare the large definitive fills from the plasma of patients on oral anticoagulation.

Table 3.4 - INR estimates for the trial fill plasmas using IRP thromboplastins

Sample	*RBT/90*		*BCT/253*		*OBT/79*	
	Lab 1	*Lab 2*	*Lab 1*	*Lab 2*	*Lab 1*	*Lab 2*
Normal plasma - A	1.0	0.9	1.0	1.0	1.0	-
Patient plasma - B	1.8	1.7	1.6	1.7	1.8	-
Patient plasma - C	3.1	3.2	2.9	3.1	2.8	-
Artificial plasma - D	3.3	3.4	2.4	2.5	2.5	-
Artificial plasma - E	5.2	6.3	3.3	3.3	3.2	-

4. PREPARATION OF THE CANDIDATE REFERENCE PLASMAS

All units of plasma used to prepare the three candidate reference materials were tested and found negative for Hepatitis B surface antigen, antibodies to Hepatitis C and antibodies to HIV 1 and 2.

4.1 Normal plasma: BCR-630 (study code A)

Plasma was collected from 18 normal healthy donors at the North London Blood Transfusion Centre, Colindale, London, United Kingdom, by conventional venepuncture into CPD-adenine anticoagulant (21 June 1995). Each unit of plasma was centrifuged twice (4200 rpm, 10 minutes, 22 ^{o}C) to remove cellular material and was buffered by the addition of N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) (0.5 mol/l, pH 7.4) to a final concentration of 20 mmol/l. Plasma units were stored overnight at 2 - 8 ^{o}C before being pooled prior to ampoule filling (22 June 1995).

4.2 Abnormal plasma-1: BCR-631 (study code B) and abnormal plasma-2: BCR 628 (study code C)

The plasma units were obtained by plasmapheresis from patients on oral anticoagulation and were purchased from Universal Reagents Inc, Indianapolis, USA. Plasma units were stored at -70 ^{o}C upon receipt (6 April 1995) before being thawed in a waterbath (37 ^{o}C) on the day of ampoule filling (abnormal plasma-1 ampouled 27 April 1995; abnormal plasma-2 ampouled 11 May 1995). Each abnormal plasma preparation required the pooling of plasma units from six patients with INR range 2 - 2.5 (Abnormal plasma-1) or 4 - 4.5 (Abnormal plasma-2). HEPES buffer (0.5 mol/l, pH 7.4) was added to each pool to give a final concentration of 20 mmol/l. The pooled plasma then underwent filtration (1.2 μm) prior to ampoule filling.

4.3 Ampoule filling

Ampoule filling, freeze-drying and secondary desiccation were carried out according to the conditions used for the preparation of International Biological Standards (6). All procedures complied with quality standard ISO 9001. All three candidate reference plasmas were filled into approximately 3,800 glass ampoules at a volume of 1.1 ml per ampoule. Filling was carried out at a temperature of 4 ^{o}C. The freeze-drying cycle of 5 days was followed by secondary desiccation over phosphorous pentoxide for 6 days before ampoule sealing and storage at -20 ^{o}C.

5. TESTING OF THE CANDIDATE REFERENCE PLASMAS

5.1 Homogeneity testing

5.1.1 Check weights during ampoule filling

The homogeneity of ampoule filling was investigated by the inclusion of check weight ampoules at every 50th position in the fill sequence. The balance used for measuring check weights was calibrated with an accuracy of ±0.1 mg for a 1.0 g standard check weight by an organisation accredited by the United Kingdom Accreditation Service (UKAS). The weight of the filled contents of 67, 77 and 73 ampoules was measured for the normal plasma preparation and for the abnormal plasma-1 and -2 respectively (Table 5.1 and Figure 5.1). The coefficient of variation for the check weights did not exceed 0.466% (with abnormal plasma-1). Examination of the check weight data for abnormal plasma-1 and -2 reveals trends to lower weights during the filling process. The extent of the check weight variability was in the range of ±1% of the mean mass. This variability was considered to be too small to require exclusion of any ampoules since experiments have shown that even a 10% change in the concentration of the normal plasma and the abnormal plasma-2 did not cause significant changes in the measured PT. The check weight variability of ±1% observed during filling would therefore be undetectable by the PT.

Table 5.1 - Check weights during ampoule filling

	Normal plasma BCR-630	*Abnormal plasma-1 BCR-631*	*Abnormal plasma-2 BCR-628*
No ampoules filled	3628	3645	3681
No of check weights	67	77	73
Mean weight (g)	1.1072	1.1119	1.1060
Range (g)	1.1027 - 1.1138	1.1013 - 1.1236	1.0975 - 1.1174
CV%	0.203	0.466	0.376
95% confidence limits of mean fill weight	±0.406%	±0.928%	±0.750%

5.1.2 Mass of the freeze-dried plasmas

The mass of the freeze-dried plasmas was estimated in six randomly selected ampoules. The mass weight was obtained by subtracting the weight of the washed and dried empty ampoule from the weight of the ampoule containing the freeze-dried material (Table 5.2). Mean weights of ampoules containing the freeze-dried material were: 6.2528 g for normal plasma (BCR-630), 6.2138 g for abnormal plasma-1 (BCR-631) and 6.2424 g for abnormal plasma-2 (BCR-628). Mean weights of the freeze-dried material were: 88.7 mg for normal plasma (BCR-630), 82.6 mg for abnormal plasma-1 (BCR-631) and 82.2 mg for abnormal plasma-2 (BCR-628). Inter-ampoule variability (CV%) was largest with the abnormal plasma-1 at 0.66%. The true inter-ampoule variability is probably less than the observed variability which also includes variability introduced during the washing and drying of the ampoules.

5.1.3 Prothrombin time tests after freeze-drying

Prothrombin time estimates were made on 20 randomly selected ampoules of each candidate plasma preparation and on 20 aliquots of a frozen pool of normal plasma. The geometric coefficient of variation of the three candidate reference plasmas did not exceed 1.92% (with abnormal plasma-2) and was actually less than the variation seen with the prothrombin times on the identical aliquots of

frozen plasma control samples in two sessions (Table 5.3). The latter estimates on identical frozen aliquots of plasma indicate that the variability of the assay method is very low (maximum geometric coefficient of variation 1.89%).

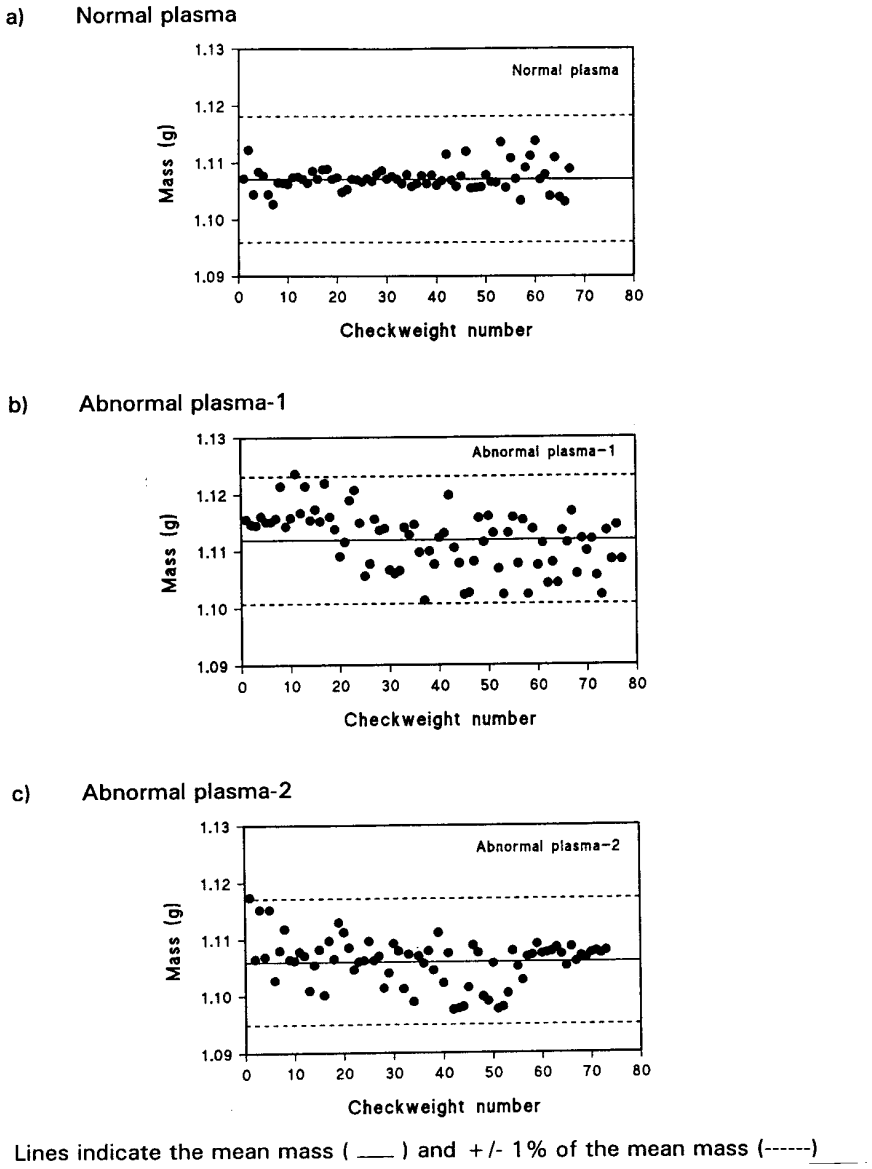

Figure 5.1 - Homogeneity testing of candidate reference plasmas. Check weights during ampoule filling.

Table 5.2 - Mass (mg) of the freeze-dried candidate plasma preparations

Sample	*Normal plasma*	*Abnormal plasma-1*	*Abnormal plasma-2*
1	89.0	81.6	82.3
2	88.8	83.2	82.1
3	88.7	82.8	82.4
4	88.3	82.8	82.3
5	88.4	82.6	82.4
6	89.0	82.5	81.5
Mean	88.7	82.6	82.2
SD	0.297	0.538	0.344
CV%	0.33	0.65	0.42

Table 5.3 - Prothrombin time estimates (seconds) after freeze-drying

Sample	*Normal Plasma*	*Frozen aliquots*	*Abnormal plasma-1*	*Frozen aliquots*	*Abnormal plasma-2*	*Frozen aliquots*
1	10.3	10.2	26.6	10.3	44.1	10.3
2	10.0	10.2	25.6	10.2	43.3	10.2
3	10.0	10.0	26.3	10.4	43.8	10.2
4	10.0	10.3	26.4	10.4	43.8	10.1
5	10.1	10.2	26.1	10.4	43.6	10.4
6	10.2	10.3	26.4	10.4	43.4	10.6
7	10.2	10.2	25.4	10.5	43.6	10.4
8	10.1	10.2	25.5	10.6	43.7	10.3
9	10.0	10.3	25.9	10.6	43.1	10.4
10	10.0	10.3	26.2	10.5	43.1	10.5
11	10.1	10.3	26.3	10.3	43.7	10.3
12	10.1	10.3	25.9	10.4	43.2	10.2
13	9.9	10.3	26.0	10.5	46.0	10.5
14	10.1	10.4	26.2	10.3	44.3	10.3
15	10.1	10.4	26.1	10.3	45.0	10.5
16	10.1	10.4	26.8	10.6	42.8	10.3
17	10.0	10.4	26.1	11.0	42.9	10.5
18	10.0	10.5	25.7	10.9	45.3	10.6
19	10.0	10.4	25.8	10.5	43.7	10.5
20	10.1	10.4	25.4	10.6	44.8	10.6
Mean PT	10.1	10.3	26	10.5	43.9	10.4
GCV%	0.92	1.1	1.5	1.89	1.92	1.45

Prothrombin times were determined for 20 ampoules of each candidate freeze-dried plasma and 20 aliquots of a frozen normal pooled plasma.

5.2 Residual moisture

Residual moisture was determined by Karl Fischer titrimetry with an uncertainty value (CV) of

5%; standardisation of the moisture meter was achieved using a check solution, provided by an organisation accredited by the United Kingdom Accreditation Service (UKAS), containing a known amount of water in methyl cellulose. Residual moisture was determined on three randomly selected ampoules from each candidate plasma preparation after freeze-drying and on another three ampoules after completion of both freeze-drying and secondary desiccation (Table 5.4). The levels of residual moisture are similar to those found with other International reference plasmas prepared under the same conditions and indicate satisfactory processing of the candidate plasmas.

Table 5.4 - Residual moisture as mass fraction (%) in the candidate plasma preparations after freeze-drying (FD only) and after both freeze-drying and secondary desiccation (FD+SD)

Sample	*Normal plasma*		*Abnormal plasma-1*		*Abnormal plasma-2*	
	BCR-630		*BCR-631*		*BCR-628*	
	FD only	*FD+SD*	*FD only*	*FD+SD*	*FD only*	*FD+SD*
1	0.7388	0.2203	1.0354	0.2174	0.7932	0.2822
2	0.7023	0.2457	1.0299	0.2308	0.7927	0.2631
3	0.7072	0.2530	1.0747	0.2070	0.7214	0.2277
Mean	0.7160	0.2397	1.0467	0.2184	0.7683	0.2577

5.3 INR values of fresh and freeze-dried candidate plasmas

The INR values of the three candidate plasmas were measured before and after freeze-drying (Table 5.5). The INR values of the fresh plasma samples underwent little change as a result of freeze-drying.

Table 5.5 - Effect of freeze-drying on INR

	Normal plasma	*Abnormal plasma-1*	*Abnormal plasma-2*
Fresh	0.96	2.4*	4.1*
Freeze-dried	0.95	2.2	3.8

Mean INR values calculated from at least 8 PT estimates with Innovin thromboplastin

* - PT estimates on plasma samples immediately after thawing and pooling

5.4 Coagulation factor levels in the freeze-dried candidate plasmas

Coagulation factors II, V, VII, X and fibrinogen together make up the enzyme cascade which is involved in the prothrombin time test. The levels of these factors were measured in the freeze-dried candidate plasmas (Table 5.6). Levels of factor V and fibrinogen were in the normal range in all three candidate plasmas. The levels of the vitamin K dependent factors, II, VII and X, were normal in the normal plasma candidate but were reduced in the abnormal plasmas as would be expected for plasma obtained from patients on oral anticoagulant therapy. The plasma with the highest INR value (abnormal plasma-2) was found to have the lowest levels of factors II, VII and X and this reflected the degree of anticoagulation. The retention of normal levels of factor V and fibrinogen in all three candidate plasmas together with the normal levels of II, VII and X in the normal plasma candidate is a good indication that freeze-drying has not had a detrimental effect on the activity of these coagulation factors.

Table 5.6 - Coagulation factor levels in the candidate plasmas

	Normal plasma BCR-630	*Abnormal plasma-1 BCR-631*	*Abnormal plasma-2 BCR-628*
Factor II (IU/ml)	0.900	0.189	0.111
	(0.858 - 0.943)	(0.184 - 0.194)	(0.108 - 0.115)
Factor V (units/ml)	1.079	1.030	1.065
	(1.010 - 1.152)	(0.988 - 1.074)	(1.001 - 1.134)
Factor VII (IU/ml)	1.031	0.413	0.182
	(0.981 - 1.085)	(0.402 - 0.425)	(0.166 - 0.199)
Factor X (IU/ml)	0.945	0.141	0.076
	(0.878 - 1.016)	(0.124 - 0.161)	(0.064 - 0.091)
Fibrinogen (g/l)	2.74	3.60	3.20
	(2.71 - 2.78)	(3.55 - 3.65)	(2.61 - 3.92)

95% confidence intervals are given in the brackets

6. STABILITY STUDIES

6.1 Accelerated degradation study

6.1.1 Design of the study

Stability of the three candidate plasmas was estimated in an accelerated degradation study which required several ampoules of each candidate plasma to be stored at elevated temperatures (+4, +20, +37, +45 ^{o}C) for various periods before measuring the PT value using the manual technique (GCV<2%, see Table 5.3). Sampling times are given in tables 6.1 and 6.6. PT estimates were carried out using two different thromboplastin reagents (Baxter Innovin and Manchester Reagent) and each sampling time involved at least 3 replicate PT estimates on each of two different ampoules to give a total of at least 6 PT estimates. Tables of PT estimates are given in Annex A. Degradation of the samples presented as an increase in the PT estimate and these were converted into INR values using the ISI values of the thromboplastin reagents. In order to analyse the data using the program of Kirkwood and Tydeman (7) it was necessary to convert the INR values into FVII concentration using a calibration curve plotting INR vs. FVII concentration. The FVII concentration of the degradation samples was then expressed as a ratio of the FVII concentration of ampoules stored at -20 ^{o}C. The predicted degradation of ampoules stored at the bulk storage temperature of -20 ^{o}C was then estimated according to the Arrhenius equation. Finally the predicted degradation rate of FVII was converted to the predicted increase in INR using the same calibration curve where loss of 50% FVII activity is equivalent to an INR increase of 1.1.

6.1.2 Results and discussion

Tables 6.1 to 6.6 give the INR values of the plasma samples stored at -20 ^{o}C and at the elevated temperatures. The predicted % loss of FVII per year for ampoules stored at -20 ^{o}C together with the equivalent predicted increase in INR value per year are shown in Table 6.7. The maximum predicted degradation (for abnormal plasma-2) would result in an increase in the INR value of 2.9×10^{-4} over one year at the storage temperature of -20 ^{o}C which is equivalent to an increase in the INR of less than 0.01%. This result indicates that the candidate plasmas are extremely stable when stored at -20 ^{o}C.

Table 6.1 - Stability of Normal plasma (BCR-630). INR values of candidate plasma samples stored at elevated temperatures determined using Baxter Innovin thromboplastin reagent.

Storage time (months)	*INR value for ampoules stored at various temperatures*				
	-20 ^{o}C	*+4 ^{o}C*	*+20 ^{o}C*	*+37 ^{o}C*	*+45 ^{o}C*
3	1.00	---	---	1.41	---
4	0.98	---	1.05	1.52	---
7	0.98	1.00	1.07	1.83	---

Table 6.2 - Stability of Abnormal plasma-1 (BCR-631). INR values of candidate plasma samples stored at elevated temperatures determined using Baxter Innovin thromboplastin reagent

Storage time (months)	*INR value for ampoules stored at various temperatures*				
	-20 °C	*+4 °C*	*+20 °C*	*+37 °C*	*+45 °C*
3	2.60	---	---	3.10	---
6	2.63	---	2.77	3.30	4.56
8.5	2.56	2.60	2.73	3.46	---

Table 6.3 - Stability of Abnormal plasma-2 (BCR-628). INR values of candidate plasma samples stored at elevated temperatures determined using Baxter Innovin thromboplastin reagent

Storage time (months)	*INR value for ampoules stored at various temperatures*				
	-20 °C	*+4 °C*	*+20 °C*	*+37 °C*	*+45 °C*
3	4.30	---	---	4.90	---
5.5	4.48	---	4.65	5.35	6.91
8	4.27	4.28	4.44	5.35	---

Each INR estimate is a mean value from at least 6 independent PT estimations

Table 6.4 - Stability of Normal plasma (BCR-630). INR values of candidate plasma samples stored at elevated temperatures determined using Manchester thromboplastin reagent.

Storage time (months)	*INR value for ampoules stored at various temperatures*				
	-20 °C	*+4 °C*	*+20 °C*	*+37 °C*	*+45 °C*
1	1.04	---	1.04	1.35	2.17
3	1.03	---	1.08	1.62	---
7	1.03	1.03	1.12	2.40	---

Table 6.5 - Stability of Abnormal plasma-1 (BCR-631). INR values of candidate plasma samples stored at elevated temperatures determined using Manchester thromboplastin reagent.

Storage time (months)	*INR value for ampoules stored at various temperatures*				
	-20 °C	*+4 °C*	*+20 °C*	*+37 °C*	*+45 °C*
3	2.74	---	3.05	3.63	5.86
5	2.91	---	3.35	4.35	6.91
8.5	2.57	2.70	2.94	4.07	---

Table 6.6 - Stability of Abnormal plasma-2 (BCR-628). INR values of candidate plasma samples stored at elevated temperatures determined using Manchester thromboplastin reagent.

Storage time (months)	*INR value for ampoules stored at various temperatures*				
	-20 °C	*+4 °C*	*+20 °C*	*+37 °C*	*+45 °C*
2.5	4.26	---	4.50	5.54	6.96
3	3.80	---	---	4.89	---
4.5	4.33	---	4.75	5.70	8.15
8	3.80	3.77	4.06	5.48	---

Each INR estimate is a mean value from at least 6 independent PT estimations.

Table 6.7 - Predicted degradation rates for candidate plasmas

Plasma sample	*Thromboplastin reagent*	*Predicted % loss of FVII per year at -20 °C*	*Predicted increase in INR per year at -20 °C*
Normal plasma A	Innovin	0.011	2.4×10^{-4}
BCR-630	Manchester	None detectable	None detectable
Abnormal plasma-1 B	Innovin	0.012	2.6×10^{-4}
BCR-631	Manchester	0.001	2.2×10^{-5}
Abnormal plasma-2 C	Innovin	0.013	2.9×10^{-4}
BCR-628	Manchester	0.001	2.2×10^{-5}

INR values of the candidate plasmas were determined using both Baxter Innovin and Manchester thromboplastin reagents.

6.2 Real-time stability study

6.2.1 Design of the study

Estimates of real-time stability were based on a comparison of prothrombin time estimates obtained in the Division of Haematology, National Institute for Biological Standards and Control, Potters Bar, United Kingdom, in November 2000 with those obtained from the same laboratory in the original calibration exercise in October 1995. Both sets of data were obtained using the same thromboplastin reagent (RBT/90) and followed the same manual methodology for the prothrombin time as described in Annex B but performed by different operators. Ampoules tested in November 2000 had been stored at -20 ^{o}C since the original calibration exercise in 1995.

6.2.2 Results and discussion

Prothrombin time estimates for the three candidate reference plasmas obtained in October 1995 and November 2000 are given in Table 6.8. Mean estimates obtained in November 2000 were shorter than the estimates obtained in 1995 with all three candidate reference plasmas and in all cases the difference was highly significant according to the unpaired t test. However, the different results obtained in November 2000 cannot be taken as evidence of instability of the preparations since,

a) the prothrombin time is an absolute measurement of a clotting time and the results are subject to operator based variation. Unavoidably, the prothrombin times from the two time-points were carried out by different operators and it is not surprising that different results were obtained. This is supported by the wide range of mean prothrombin time estimates, obtained using RBT/90, in the original calibration exercise by the different laboratories, (Tables 7.2, 7.3, 7.4) e.g. from 16.1 to 19.7 seconds for the normal plasma (BCR-630), from 35.7 to 47.1 seconds for the abnormal plasma-1 (BCR-631) and from 55.8 to 84.3 seconds for the abnormal plasma-2 (BCR-628). These wide ranges were obtained despite the use of the same thromboplastin reagent and manual technique and emphasise the operator-induced variability. It is therefore impossible to determine whether the difference in results between the two time-points reflects instability or operator variability.

b) the prothrombin times obtained in November 2000 are shorter than those obtained in October 1995. Instability of the reference plasmas would be expected to lead to a prolongation of the prothrombin time as seen with the accelerated degradation study. The shorter times obtained in November 2000 are more likely a reflection of operator variability, particularly since the same trend was found with all three reference plasmas.

Table 6.8 - Comparison of prothrombin time estimates (seconds) obtained in November 2000 with estimates obtained in the original calibration exercise in October 1995.

Ampoule	*Normal plasma*		*Abnormal plasma-1*		*Abnormal plasma-2*	
	BCR-630		*BCR-631*		*BCR-628*	
	1995	*2000*	*1995*	*2000*	*1995*	*2000*
1	19.3	18.3	43.6	39.9	74.5	70.8
2	19.1	18.5	43.6	40.7	71.4	68.3
3	18.6	19.3	43.2	39.9	74.3	68.7
4	18.6	18.8	41.8	40.1	71.9	68.1
5	19.4	17.9	44.0	39.9	73.6	68.1
6	20.1	19.0	42.5	40.1	69.3	67.9
7	19.1	19.5	45.6	38.7	76.2	69.2
8	19.1	18.2	42.8	39.7	72.7	68.4
9	19.4	17.4	41.8	39.7	78.5	68.5
10	19.5	17.8	44.7	39.3	74.9	69.4
Mean	19.2	18.5	43.3	39.8	73.7	68.7
95% conf limits	18.9 - 19.5	18.0 - 19.0	42.5 - 44.2	39.4 - 40.2	71.9 - 75.6	68.1 - 69.4
GCV%	2.30	3.75	2.83	1.34	3.58	1.26
p value	<0.01		<0.0001		<0.0001	

p values obtained using the unpaired t test

The conclusions which can be drawn from this real-time study are therefore very limited and not quantifiable. It can only be stated that the mean prothrombin time estimates obtained in November 2000 are within the range of the mean laboratory estimates obtained in the original calibration exercise for all three candidate reference plasmas and do not provide evidence of instability. It is proposed that further real-time stability studies for the prothrombin time should be designed using automated coagulometers in order to avoid the operator variability. These studies should be carried out at regular intervals during the lifetime of the reference plasmas.

6.3 Storage

The three candidate reference plasmas are stored at -20 ^{o}C. Experience in the production of plasma standards over many years and the results of the accelerated degradation study in section 6.1 have indicated that this temperature is suitable for the long-term storage of freeze-dried plasma reference preparations.

7. CERTIFICATION MEASUREMENTS

7.1 The collaborative study

The INR calibration of the three candidate reference plasmas was carried out as part of the same exercise in which the candidate replacement International Reference Preparation for Thromboplastin, Human, Plain was calibrated (see study protocol in Annex B). The considerable degree of overlap in the requirements of the two exercises (e.g. determination of MNPT from 20 normal individuals) and the need to conserve stocks of the International Reference Thromboplastin preparations were the main reasons for combining the two calibration exercises.

The study was carried out in ten assay sessions, which were identical except for variation in the order in which the different thromboplastin reagents were used. All prothrombin time estimates were obtained using the manual method. In each session single prothrombin time estimates were carried out on the three candidate reference plasmas and two fresh normal plasma samples (one male and one female) using the six different thromboplastin reagents described below:

- BCT/253 (IRP Human, plain) ISI 1.1;
- RBT/90 (IRP Rabbit, plain) ISI 1.0;
- OBT/79 (IRP Bovine, combined) ISI 1.0;
- X/95 (candidate IRP, Human recombinant, plain) ISI 0.941*;
- Y/95 (candidate IRP, Human recombinant, plain) ISI 1.004*;
- PL (commercial human placental reagent) ISI 1.165*.

*- ISI values calculated from results in the current study

The fresh normal plasma samples were obtained from 2 different donors in each of the 10 assay sessions. Prothrombin time estimates from the total of 20 normal plasma samples were used to calculate the mean normal prothrombin time (MNPT) which was used in the calculation of INR. Fresh ampoules of the three candidate plasmas were used in each session. The order of using the thromboplastin reagents was specified on the results sheets. Raw data (prothrombin times) were returned to NIBSC for analysis. Only the prothrombin time results obtained using the International Reference Thromboplastins (BCT/253, RBT/90, OBT/79) were used for INR calibration of the candidate plasmas. The results of the collaborative study for the INR calibration of the candidate reference plasmas have been published (8).

7.2 Analytical methods

7.2.1 Prothrombin time methodology

All participants used the manual version of the prothrombin time test as described in the study protocol (Annex B) which also supplied comprehensive instructions regarding collection of the fresh plasma samples and reconstitution of the thromboplastin reagents and the freeze-dried candidate reference plasmas.

7.2.2 Calculation of INR estimates and variability

Mean normal prothrombin times (MNPT) were calculated for each thromboplastin reagent in each laboratory by taking the geometric mean of the PT values of the 20 fresh normal plasma

$$INR = \left(\frac{\text{mean candidate plasma PT}}{} \right)^{ISI}$$

samples. Prothrombin times for the candidate plasmas A, B and C were calculated as geometric means of the 10 sessions for each laboratory and each reagent. INR estimates were calculated, for each laboratory and each reagent, according to the following equation:

where ISI is the International Sensitivity Index of the thromboplastin reagent.

INR estimates were combined across laboratories by taking the geometric mean value. Estimates of intra- and inter-laboratory variability are expressed as the geometric coefficient of variation (GCV%) (2).

7.3 Results of the calibration exercise

All of the results received in the study are presented. However, the results obtained by laboratory 13 using thromboplastin OBT/79 with all three candidate plasmas have not been included in the calculation of the overall mean values since this laboratory reported problems in determining the end-point of clot formation.

Although the results obtained using all six thromboplastin reagents are given, only the results from the three International Reference Thromboplastins (BCT/253, RBT/90, OBT/79) were used for the INR calibration of the three candidate plasmas.

7.3.1 Mean normal prothrombin times

The MNPT values for each laboratory with the six thromboplastin reagents are given in Table 7.1. Inter-laboratory variability ranged from a GCV of 4.9% with thromboplastin BCT/253 (MNPT range 12.6 to 16.1 seconds) to 12.6% with thromboplastin OBT/79 (MNPT range 33.0 to 42.2 seconds).

Table 7.1 – Mean normal prothrombin times (seconds) for each laboratory

LAB	*BCT/253*	*OBT/79*	*RBT/90*	*X/95*	*Y/95*	*PL*
1	14.4	35.2	18.9	12.3	10.7	13.2
2	16.1	41.1	20.0	16.0	14.9	16.4
3	14.5	36.1	18.1	13.8	11.2	14.5
4	14.1	37.8	18.7	13.0	10.7	14.4
5	14.4	40.2	17.4	14.0	11.7	14.7
6	14.6	35.4	18.5	12.6	10.4	13.6
7	14.3	42.2	17.6	14.4	12.6	13.8
8	14.4	35.2	18.4	12.6	10.6	14.3
9	14.4	37.7	18.4	13.4	12.0	14.5
10	14.0	36.6	18.0	12.9	10.9	13.8
11	14.0	35.3	17.5	13.1	10.7	13.1
12	13.6	35.1	16.6	12.2	9.9	13.3
13	13.8	33.8	16.9	11.6	10.1	12.2
14	14.0	34.4	18.8	12.4	10.4	13.0
15	14.5	37.9	18.9	13.5	11.4	14.4
16	15.0	38.1	18.7	13.4	11.2	14.6

LAB	*BCT/253*	*OBT/79*	*RBT/90*	*X/95*	*Y/95*	*PL*
17	13.9	35.0	17.7	13.9	11.5	14.5
18	14.0	33.0	16.7	12.5	10.7	13.0
19	12.6	34.6	16.6	11.8	9.8	12.8
20	14.7	36.6	19.0	14.8	12.2	15.6
Geometric Mean	14.5	36.1	17.5	14.2	11.8	14.8
GCV (%)	4.9	12.6	5.4	6.5	9.0	6.2

Each MNPT represents the geometric mean value from single prothrombin times carried out on twenty fresh normal plasma samples.

7.3.2 Prothrombin time estimates for candidate reference plasmas

7.3.2.1 Normal plasma (coded A) BCR-630

The geometric means of the 10 PT estimates for the normal plasma (A) from each laboratory, using the six thromboplastin reagents, are given in Table 7.2. Mean PT estimates ranged from 13.2 to 16.1 seconds for BCT/253; 32.6 to 40.7 seconds for OBT/79 (excluding the outlying value for laboratory 13); 16.1 to 19.7 seconds for RBT/90; 13.1 to 16.2 seconds for X/95; 10.7 to 15.5 seconds for Y/95 and from 13.5 to 16.5 seconds for PL.

7.3.2.2 Abnormal plasma-1 (coded B) BCR-631

The geometric means of the 10 PT estimates for the abnormal plasma-1 (B) from each laboratory, using the six thromboplastin reagents, are given in Table 7.3. Mean PT estimates ranged from 29.1 to 36.9 seconds for BCT/253; 69.9 to 86.6 seconds for OBT/79; 35.7 to 47.1 seconds for RBT/90; 31.9 to 38.3 seconds for X/95; 27.5 to 36.7 seconds for Y/95 and from 26.4 to 36.6 seconds for PL.

7.3.2.3 Abnormal plasma-2 (coded C) BCR-628

The geometric means of the 10 PT estimates for the abnormal plasma-2 (C) from each laboratory, using the six thromboplastin reagents, are given in Table 7.4. Mean PT estimates ranged from 40.8 to 58.2 seconds for BCT/253; 91.9 to 129.0 seconds for OBT/79; 55.8 to 84.3 seconds for RBT/90; 42.2 to 56.6 seconds for X/95; 43.2 to 56.7 seconds for Y/95 and from 37.2 to 52.1 seconds for PL.

7.3.3 Intra-laboratory variability of prothrombin time estimates

Intra-laboratory variability has been expressed as the GCV% of the PT estimates for the three candidate plasmas from the 10 assay sessions. Intra-laboratory variability for PT estimates of the normal plasma (A) are given in Table 7.5; these ranged from a GCV of 1.2% (lab 1, OBT/79) to 14.1% (lab 12, X/95). Intra-laboratory variability for PT estimates of abnormal plasma-1 (B) are given in Table 7.6; these ranged from 1.0% (lab 19, OBT/79) to 20.8 (lab 13, OBT/79). Intra-laboratory variability for PT estimates of abnormal plasma-2 (C) are given in Table 7.7; these ranged from 0.6% (lab 1, PL) to 16.0% (lab 13, OBT/79).

Table 7.2 - Mean PT estimates (seconds) for the normal plasma BCR-630

LAB	*BCT/253*	*OBT/79*	*RBT/90*	*X/95*	*Y/95*	*PL*
1	14.0	33.7	17.7	13.3	11.4	13.9
2	16.1	39.8	19.7	16.2	15.5	16.5
3	14.5	35.5	17.8	14.7	11.4	15.6
4	13.9	35.4	17.0	14.2	11.4	15.0
5	15.2	40.7	16.9	14.9	12.6	16.1
6	14.2	33.3	17.7	13.1	11.0	14.1
7	14.5	40.1	17.0	15.0	13.2	15.2
8	14.3	34.3	17.1	13.5	10.9	14.9
9	14.7	35.6	18.3	14.7	12.7	15.3
10	14.4	35.2	17.1	13.9	11.5	14.9
11	13.2	33.3	16.9	13.7	11.9	14.4
12	14.3	33.4	16.3	14.4	10.7	14.3
13	15.0	55.2	17.3	13.3	11.8	13.7
14	14.9	33.6	18.4	13.6	11.4	13.9
15	15.0	36.2	18.8	14.7	11.9	15.2
16	15.0	35.1	17.4	13.6	11.6	15.1
17	14.5	33.6	17.6	14.7	12.1	15.1
18	14.2	34.1	17.0	13.3	11.2	13.6
19	13.3	32.6	16.1	13.1	10.7	13.5
20	15.6	37.1	19.2	15.9	12.6	16.4

Results are the geometric means of 10 PT estimates (seconds) carried out on different ampoules

Table 7.3 - Mean PT estimates (seconds) for the abnormal plasma-1 BCR-631

LAB	*BCT/253*	*OBT/79*	*RBT/90*	*X/95*	*Y/95*	*PL*
1	33.4	78.4	43.1	32.3	30.6	31.1
2	34.6	85.3	45.3	38.3	36.7	36.3
3	30.7	83.7	42.4	33.4	28.8	34.9
4	30.7	80.0	39.6	34.3	28.6	35.6
5	29.1	75.5	35.7	33.3	28.6	33.2
6	32.5	78.2	41.9	32.6	28.1	33.5
7	34.8	86.6	43.4	36.5	36.3	36.3
8	29.2	78.5	39.2	33.8	27.5	34.1
9	34.1	82.9	43.0	37.7	30.2	36.6
10	30.2	81.0	39.9	33.1	29.8	33.6
11	32.0	80.3	47.1	35.1	28.8	34.4
12	32.0	78.0	39.5	32.4	29.6	33.7
13	32.4	69.9	40.5	28.7	29.4	26.4
14	36.9	79.2	43.8	31.9	28.0	33.7
15	32.3	85.9	45.3	36.2	30.2	35.6
16	31.0	83.2	39.9	33.3	28.8	34.2
17	32.1	81.0	44.4	36.3	29.3	35.5
18	31.4	81.2	38.5	34.8	29.1	34.1
19	32.9	77.0	40.8	32.6	31.6	34.4
20	34.0	82.8	43.3	35.0	29.4	35.8

Results are the geometric means of 10 PT estimates (seconds) carried out on different ampoules.

Table 7.4 - Mean PT estimates (seconds) for the abnormal plasma-2 BCR-628

LAB	*BCT/253*	*OBT/79*	*RBT/90*	*X/95*	*Y/95*	*PL*
1	52.4	116.3	78.1	47.9	47.8	43.1
2	55.5	123.5	84.3	56.6	55.4	52.1
3	43.2	121.7	74.1	48.1	47.3	47.3
4	44.1	113.6	68.9	50.9	45.1	48.6
5	40.8	110.2	55.8	46.4	43.7	44.9
6	47.7	117.8	75.1	49.3	47.0	48.5
7	52.8	129.0	80.8	54.1	56.7	49.9
8	42.8	121.2	70.0	49.5	43.2	49.3
9	49.0	116.1	69.2	53.5	48.4	50.5
10	44.0	116.8	69.2	48.8	49.1	46.8
11	48.2	113.2	75.7	49.4	47.0	46.6
12	44.0	112.2	67.4	46.6	49.5	46.3
13	47.0	91.9	57.3	42.2	46.0	37.2

LAB	BCT/253	OBT/79	RBT/90	X/95	Y/95	PL
14	58.2	116.8	76.9	47.1	45.4	46.3
15	46.2	121.7	81.4	52.6	48.9	48.1
16	44.5	118.3	70.1	48.5	45.7	47.0
17	45.4	117.4	77.8	53.5	47.2	48.4
18	47.1	114.4	62.8	49.9	48.4	48.6
19	49.0	114.7	68.0	46.5	49.5	47.7
20	49.7	117.3	73.7	50.1	47.9	50.3

Results are the geometric means of 10 PT estimates (seconds) carried out on different ampoules.

Table 7.5 - Intra-laboratory variability (GCV%) of PT estimates for the normal plasma

LAB	BCT/253	OBT/79	RBT/90	X/95	Y/95	PL
1	2.6	1.2	1.7	2.9	3.4	2.1
2	4.2	2.4	2.4	2.9	4.1	3.3
3	2.2	1.7	2.5	2.6	1.8	3.6
4	9.3	5.6	4.6	4.8	10.1	6.9
5	2.8	5.2	3.0	1.6	5.2	3.1
6	3.8	2.7	4.2	3.5	2.6	2.9
7	4.9	4.3	7.8	3.9	5.5	1.7
8	5.0	4.0	7.2	8.4	10.7	6.0
9	5.5	6.8	4.9	5.9	7.9	7.8
10	2.8	3.5	4.3	4.4	3.8	2.3
11	3.3	9.0	11.8	5.3	11.9	7.8
12	7.9	2.5	6.2	14.1	5.9	5.6
13	4.9	10.0	7.5	4.2	4.1	5.6
14	4.2	2.0	2.5	3.3	4.3	4.9
15	8.9	2.3	5.6	5.1	3.0	3.8
16	9.9	8.0	4.5	7.3	14.0	11.6
17	3.1	3.9	3.1	5.0	3.7	4.3
18	4.3	6.4	3.7	8.7	9.3	7.3
19	2.2	2.0	1.7	3.2	4.9	2.1
20	4.0	2.1	2.3	2.9	8.1	7.0

Table 7.6 - Intra-laboratory variability (GCV%) of PT estimates for the abnormal plasma-1

LAB	BCT/253	OBT/79	RBT/90	X/95	Y/95	PL
1	3.5	2.0	2.4	2.0	3.8	3.2
2	3.9	3.0	6.4	4.3	3.2	3.1
3	2.7	1.3	3.0	1.9	3.0	2.9

4	6.2	4.6	9.9	9.3	7.2	5.0
5	2.7	2.2	4.7	2.8	3.7	4.2
6	2.2	2.4	2.4	2.0	2.5	2.9
7	4.4	4.8	13.9	4.0	8.3	2.7
8	7.0	6.1	3.5	7.2	9.3	3.6
9	5.1	5.7	8.8	7.4	7.8	4.1
10	3.0	2.1	3.7	4.1	3.6	4.6
11	10.3	7.8	16.9	5.0	3.4	4.0
12	7.3	2.4	8.5	6.2	6.0	2.9
13	10.7	20.8	13.4	10.1	9.3	10.2
14	4.1	3.0	3.6	3.5	4.9	2.7
15	5.7	4.8	5.9	3.4	7.0	6.6
16	4.3	5.9	4.7	5.0	6.6	7.8
17	3.7	7.5	4.7	3.2	5.0	5.6
18	4.0	2.8	4.6	4.7	7.6	12.7
19	2.6	1.0	4.3	3.6	6.1	4.2
20	1.5	3.3	2.8	2.9	3.8	4.6

Table 7.7 - Intra-laboratory variability (GCV%) of PT estimates for the abnormal plasma-2

LAB	*BCT/253*	*OBT/79*	*RBT/90*	*X/95*	*Y/95*	*PL*
1	5.3	2.2	5.7	3.6	4.5	0.6
2	4.3	3.1	2.5	3.6	4.2	3.8
3	2.8	2.1	3.4	2.3	2.4	2.1
4	8.9	2.8	15.1	8.0	6.0	4.3
5	3.8	2.8	4.8	2.9	3.7	5.2
6	2.4	2.6	1.9	2.8	1.7	4.0
7	6.7	3.1	6.8	6.1	7.5	1.6
8	8.4	2.7	7.3	4.3	5.6	6.7
9	7.4	4.7	14.3	6.0	5.1	6.3
10	4.0	2.4	3.5	2.6	3.8	2.9
11	14.5	10.3	8.2	6.6	5.8	4.2
12	7.1	2.3	7.8	4.3	6.7	4.4
13	8.2	16.0	8.8	9.0	7.9	7.9
14	6.3	2.0	8.4	3.6	2.6	3.4
15	6.4	3.6	10.2	5.1	6.7	2.4
16	5.2	3.9	11.0	4.9	6.7	8.5
17	5.1	7.9	6.5	5.0	3.7	6.1
18	3.7	4.5	8.8	3.5	4.3	6.1
19	3.4	2.2	3.9	2.8	4.6	2.4
20	3.8	3.0	3.6	2.2	3.5	8.0

7.3.4 INR estimates for candidate reference plasmas

7.3.4.1 Normal plasma (coded A) BCR-630

The mean INR estimates for the normal plasma (A) from each laboratory using the six thromboplastin reagents are given in Table 7.8 together with the overall combined INR values and estimates of inter-laboratory variability (GCV%). The results with the three International Reference Thromboplastins are also shown in Figure 7.1. INR estimates ranged from 0.94 to 1.09 for BCT/253; 0.92 to 1.03 for OBT/79 (excluding the outlying value of 1.63); 0.91 to 1.02 for RBT/90; 1.01 to 1.17 for X/95; 1.02 to 1.16 for Y/95; 1.01 to 1.14 for PL. Inter-laboratory variability was low with GCV's below 4.5% for INR estimates obtained with all thromboplastin reagents. Overall combined (geometric mean) INR estimates ranged from 0.96 (OBT/79) to 1.07 (X/95, PL).

7.3.4.2 Abnormal plasma-1 (coded B) BCR-631

The mean INR estimates for abnormal plasma-1 (B) from each laboratory using the six thromboplastin reagents are given in Table 7.9 together with the overall combined INR values and estimates of inter-laboratory variability (GCV%). The results with the three International Reference Thromboplastins are also shown in Figure 7.2. INR estimates ranged from 2.17 to 2.90 for BCT/253; 1.99 to 2.46 for OBT/79; 2.05 to 2.68 for RBT/90; 2.24 to 2.64 for X/95; 2.43 to 3.25 for Y/95; 2.45 to 3.17 for PL. Inter-laboratory variability ranged from a GCV of 5.07% (X/95) to 8.37% (BCT/253). Overall combined (geometric mean) INR estimates ranged from 2.21 (OBT/79) to 2.83 (PL).

7.3.4.3 Abnormal plasma-2 (coded C) BCR-628

The mean INR estimates for abnormal plasma-2 (C) from each laboratory using the six thromboplastin reagents are given in Table 7.10 together with the overall combined INR values and estimates of inter-laboratory variability (GCV%). The results with the three International Reference Thromboplastins are also shown in Figure 7.3. INR estimates ranged from 3.14 to 4.78 for BCT/253; 2.72 to 3.47 for OBT/79; 3.21 to 4.39 for RBT/90; 3.08 to 3.69 for X/95; 3.74 to 5.10 for Y/95; 3.65 to 4.64 for PL. Inter-laboratory variability ranged from a GCV of 5.36% (X/95) to 10.99% (BCT/253). Overall combined (geometric mean) INR estimates ranged from 3.20 (OBT/79) to 4.33 (Y/95).

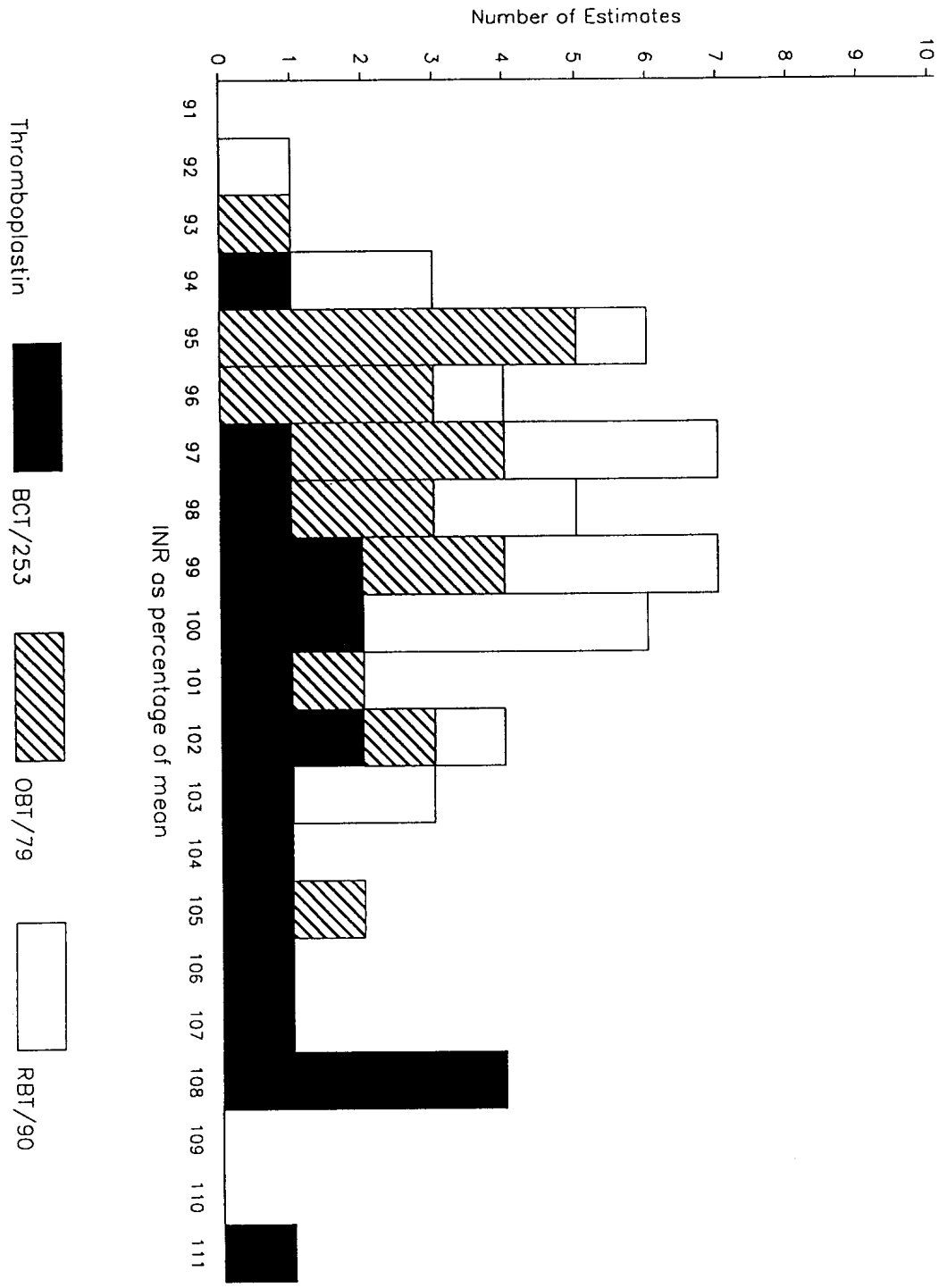

Figure 7.1 - INR values for Normal plasma (A) BCR-630 from individual laboratories expressed as a percentage of the mean INR

Table 7.8 - INR estimates for the normal plasma (A) BCR-630

LAB	*BCT/253*	*OBT/79*	*RBT/90*	*X/95*	*Y/95*	*PL*
1	0.97	0.96	0.94	1.07	1.07	1.07
2	1.00	0.97	0.99	1.01	1.04	1.01
3	0.98	0.98	0.98	1.06	1.02	1.08
4	0.98	0.94	0.91	1.09	1.06	1.05
5	1.06	1.01	0.97	1.06	1.07	1.11
6	0.96	0.94	0.96	1.04	1.06	1.04
7	1.01	0.95	0.97	1.04	1.05	1.12
8	0.99	0.97	0.93	1.07	1.03	1.05
9	1.02	0.94	0.99	1.09	1.06	1.07
10	1.03	0.96	0.95	1.07	1.05	1.09
11	0.94	0.94	0.96	1.04	1.11	1.12
12	1.06	0.95	0.98	1.17	1.08	1.09
13	1.09	1.63*	1.02	1.14	1.16	1.14
14	1.07	0.98	0.98	1.09	1.10	1.08
15	1.04	0.95	0.99	1.08	1.05	1.06
16	0.99	0.92	0.93	1.02	1.04	1.04
17	1.05	0.96	0.99	1.05	1.06	1.05
18	1.01	1.03	1.02	1.06	1.05	1.05
19	1.06	0.94	0.97	1.10	1.09	1.07
20	1.07	1.01	1.01	1.06	1.04	1.06
Mean INR	1.02	0.96	0.97	1.07	1.06	1.07
Interlab GCV (%)	4.32	3.05	3.27	3.46	3.01	3.06

* the result for OBT/79 for lab 13 is excluded from the mean and GCV calculations

Each INR estimate is derived from the mean of 10 PT determinations.

Table 7.9 - INR estimates for the abnormal plasma-1 (B) BCR-631

LAB	*BCT/253*	*OBT/79*	*RBT/90*	*X/95*	*Y/95*	*PL*
1	2.52	2.23	2.28	2.49	2.88	2.71
2	2.31	2.07	2.27	2.27	2.47	2.52
3	2.29	2.32	2.34	2.29	2.59	2.77
4	2.35	2.12	2.11	2.49	2.68	2.88
5	2.17	1.99	2.05	2.26	2.45	2.59
6	2.41	2.21	2.26	2.45	2.72	2.85
7	2.66	2.05	2.46	2.39	2.90	3.10
8	2.17	2.23	2.13	2.54	2.60	2.76
9	2.58	2.20	2.33	2.64	2.54	2.94
10	2.32	2.21	2.21	2.42	2.74	2.81
11	2.48	2.28	2.68	2.52	2.71	3.08
12	2.57	2.22	2.37	2.51	3.01	2.95
13	2.55	2.07*	2.40	2.35	2.91	2.45
14	2.90	2.30	2.33	2.43	2.71	3.03

LAB	*BCT/253*	*OBT/79*	*RBT/90*	*X/95*	*Y/95*	*PL*
15	2.41	2.26	2.40	2.52	2.66	2.88
16	2.22	2.18	2.13	2.36	2.58	2.69
17	2.52	2.31	2.50	2.46	2.56	2.84
18	2.44	2.46	2.30	2.63	2.72	3.07
19	2.88	2.22	2.46	2.60	3.25	3.17
20	2.51	2.26	2.28	2.24	2.43	2.64
Mean INR	2.45	2.21	2.31	2.44	2.70	2.83
Interlab GCV (%)	8.37	5.85	6.65	5.07	7.58	7.33

* the result for OBT/79 for lab 13 is excluded from the mean and GCV calculations

Each INR estimate is derived from the mean of 10 PT determinations.

Table 7.10 - INR estimates for the abnormal plasma-2 (C) BCR-628

LAB	*BCT/253*	*OBT/79*	*RBT/90*	*X/95*	*Y/95*	*PL*
1	4.14	3.31	4.13	3.60	4.50	3.97
2	3.89	3.00	4.22	3.28	3.74	3.84
3	3.33	3.37	4.09	3.23	4.26	3.95
4	3.50	3.01	3.67	3.60	4.23	4.14
5	3.14	2.74	3.21	3.08	3.74	3.68
6	3.66	3.32	4.05	3.62	4.56	4.38
7	4.20	3.06	4.08	3.46	4.54	4.49
8	3.31	3.44	3.81	3.64	4.10	4.23
9	3.84	3.08	3.75	3.67	4.07	4.28
10	3.52	3.19	3.84	3.49	4.53	4.14
11	3.89	3.21	4.32	3.48	4.42	4.39
12	3.65	3.20	4.05	3.53	5.05	4.27
13	3.84	2.72*	3.40	3.38	4.56	3.65
14	4.78	3.39	4.09	3.50	4.40	4.39
15	3.58	3.21	4.31	3.59	4.31	4.09
16	3.30	3.10	3.74	3.36	4.10	3.90
17	3.69	3.36	4.39	3.54	4.15	4.08
18	3.80	3.47	3.76	3.69	4.54	4.63
19	4.46	3.31	4.09	3.63	5.10	4.64
20	3.82	3.21	3.88	3.14	3.96	3.92
Mean INR	3.75	3.20	3.93	3.47	4.33	4.14
Interlab GCV (%)	10.99	5.95	8.29	5.36	8.53	7.16

* the result for OBT/79 for lab 13 is excluded from the mean and GCV calculations

Each INR estimate is derived from the mean of 10 PT determinations.

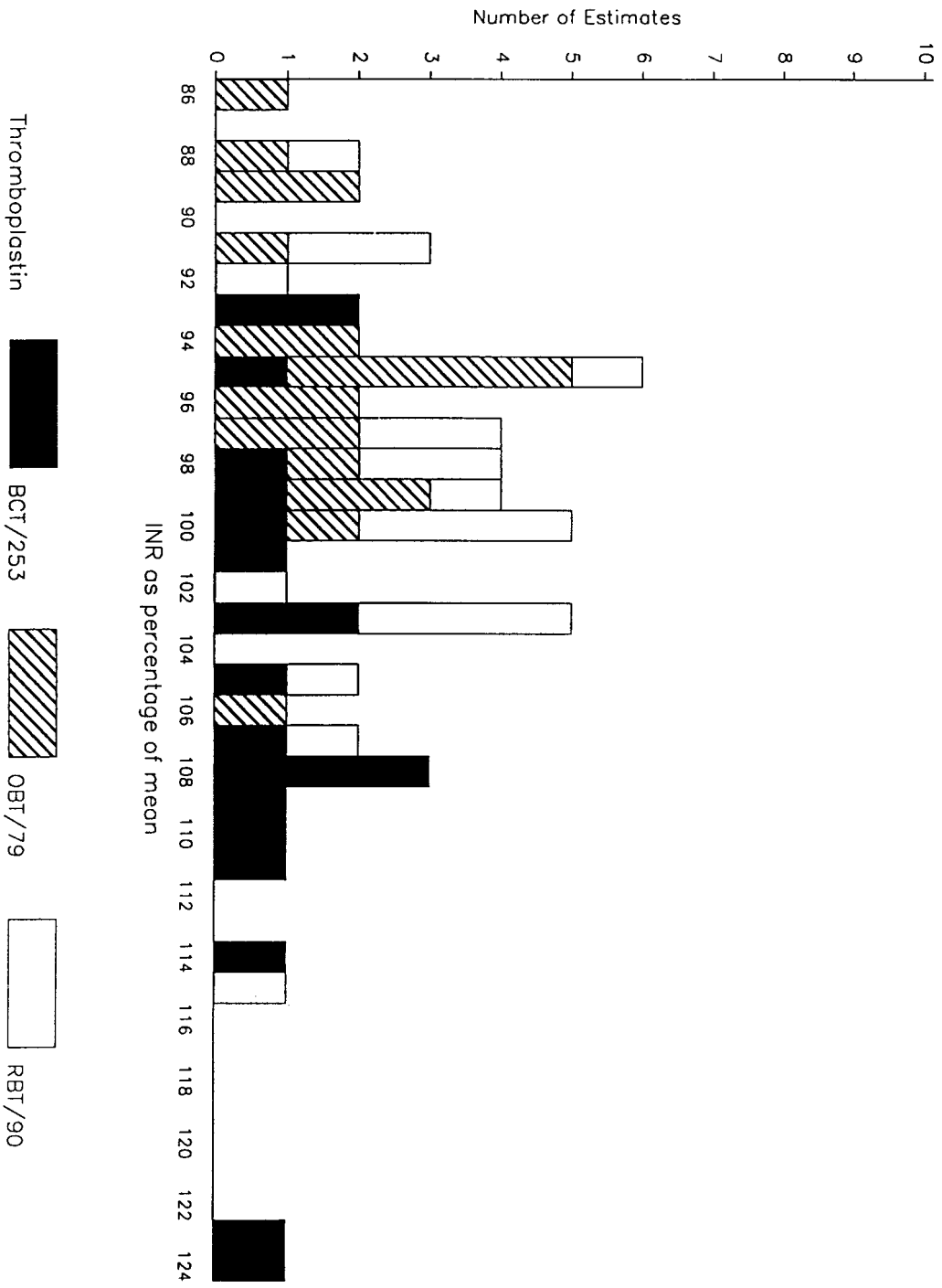

Figure 7.2 - INR values for abnormal plasma-1 (B) BCR-631 from individual laboratories expressed as a percentage of the mean INR.

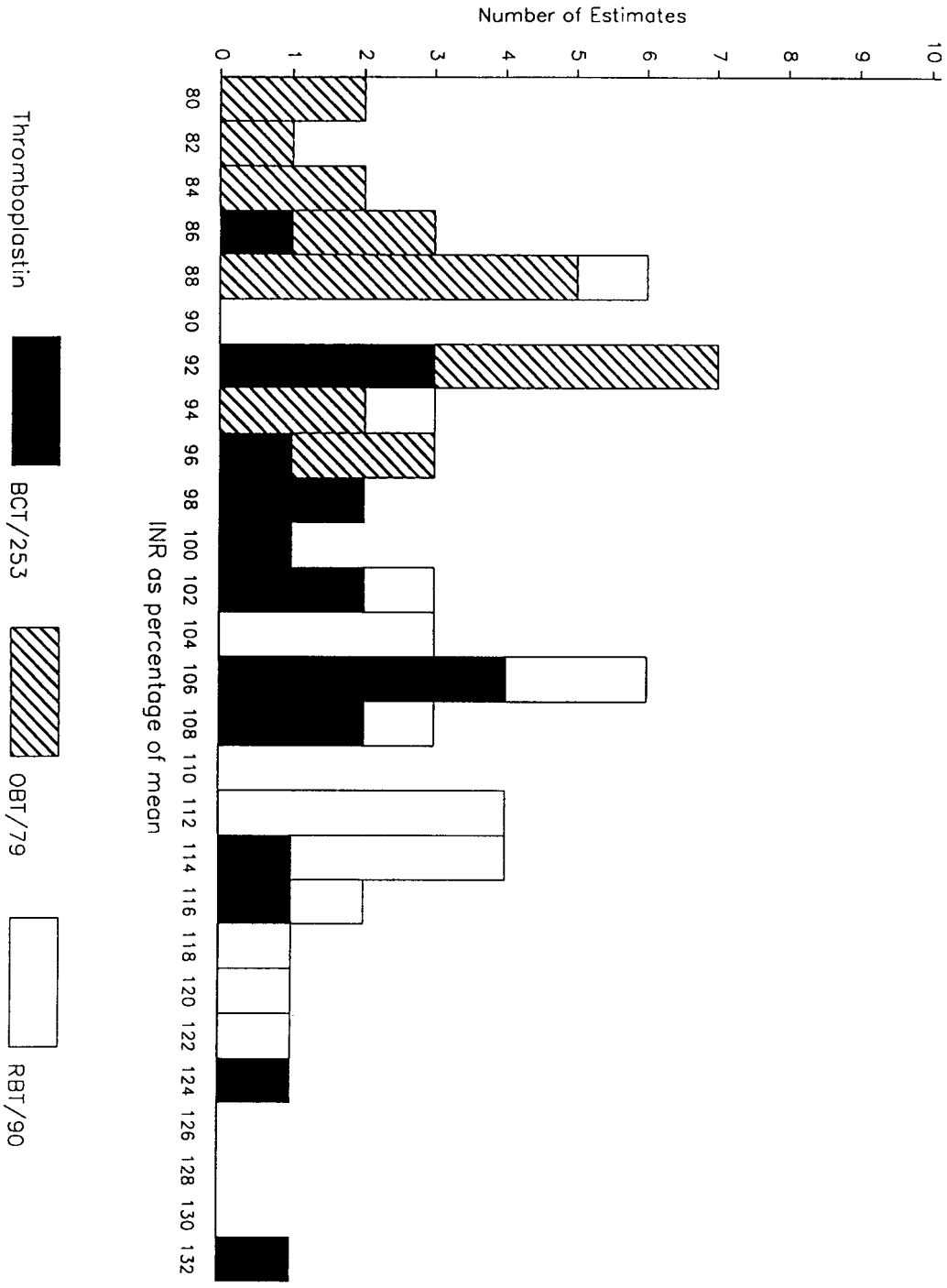

Figure 7.3 - INR values for abnormal plasma-2 (C) BCR-628 from individual laboratories expressed as a percentage of the mean INR.

7.3.5 INR calibration of candidate reference plasmas

Table 7.11 summarises the combined (geometric mean) INR estimates for the three candidate plasmas obtained with each of the three International Reference Thromboplastins (BCT/253; OBT/79; RBT/90). For each candidate plasma some statistically significant differences were found between the combined (geometric mean) INR estimates obtained with the different thromboplastins. The significant differences found with the normal plasma (A) and abnormal plasma-1 (B) are largely caused by the extremely good inter-laboratory agreement in INR estimates with each thromboplastin reagent. With the abnormal plasma-2 (C) the significant differences in INR values between BCT/253 and OBT/79 and between RBT/90 and OBT/79 are more obvious with OBT/79 clearly giving a lower INR value.

Table 7.11 - Combined (geometric mean) INR values for the candidate reference plasmas using the International Reference Thromboplastins

Plasma	*Thromboplastin*	*Geometric mean INR values*	*GCV%*
Normal plasma (A)	BCT/253	1.02	4.32
BCR-630	RBT/90	0.97	3.27
	OBT/79	0.96	3.05
Abnormal plasma-1 (B)	BCT/253	2.45	8.37
BCR-631	RBT/90	2.31	6.65
	OBT/79	2.21	5.85
Abnormal plasma-2 (C)	BCT/253	3.75	10.99
BCR-628	RBT/90	3.93	8.29
	OBT/79	3.20	5.95

Geometric mean INR values were calculated from the results of all laboratories excluding those from laboratory 13 obtained with thromboplastin OBT/79.

Statistically significant differences between geometric mean INR values were as follows:

Normal plasma (A)	BCT/253 and OBT/79	$p<0.0001$
	BCT/253 and RBT/90	$p<0.0001$
Abnormal plasma-1 (B)	BCT/253 and OBT/79	$p<0.0001$
	BCT/253 and RBT/90	$p<0.006$
Abnormal plasma-2 (C)	BCT/253 and OBT/79	$p<0.0001$
	OBT/79 and RBT/90	$p<0.0001$

7.4 Technical discussion of the results

All participants received a report on the certification exercise and were invited to comment on the acceptability of results and the assignment of INR. The following conclusions were agreed.

7.4.1 Acceptability of results

All results were accepted for inclusion in the study except for the results from laboratory 13, using OBT/79, since they reported difficulties in detecting the clotting end-point when using this thromboplastin reagent.

7.4.2 Assignment of INR to the candidate reference plasmas

Three options were considered:

- geometric mean of results from all three International Reference Thromboplastin reagents,
- geometric mean of results from BCT/253 and RBT/90,
- three different INR values assigned to each reference plasma corresponding to the results obtained with the three International Reference Thromboplastin reagents.

The large discrepancy between the mean INR obtained with OBT/79 and the other two thromboplastin reagents, for abnormal plasma-2, ruled out option 1. However, the small differences in mean INR obtained between BCT/253 and RBT/90 for all three candidate plasmas allowed option 2 to be accepted. The assigned values are intended to be valid for use with any thromboplastin of human or rabbit origin.

It was agreed that the INR values obtained using OBT/79 would not be formally assigned but would be made available in the "Certificates of Analysis" which accompany the reference materials.

8. CERTIFIED VALUES AND UNCERTAINTIES

The reference plasmas have been assigned the following INR values (obtained by calculating the geometric mean of results obtained with BCT/253 and RBT/90):

BCR No	*Material*	*INR value*[1)]	*Uncertainty*[2)]	*No of accepted results*
630	Normal plasma	0.99	0.02	40
631	Abnormal plasma-1	2.38	0.06	40
628	Abnormal plasma-2	3.84	0.12	40

1) These values are the geometric means from 40 independent estimates

2) These values are the half width of the 95% confidence intervals of the mean INR values in 1)

9. REFERENCES

1) Chanarin I. (1989) Laboratory Haematology. An account of laboratory techniques. Churchill Livingstone.

2) Kirkwood TBL. (1979) Geometric means and measures of dispersion. Biometrics **35**, 908 - 909.

3) World Health Organization (1999) Guidelines for thromboplastins and plasma used to control oral anticoagulant therapy. WHO Technical Report Series No **889**, 64 - 93.

4) Barrowcliffe TW and Thomas DP (1985) The provision of standards for blood coagulation factors. Recent Advances in Blood Coagulation (Poller, L. ed.) Churchill Livingstone. **4**, 251 - 266.

5) Hubbard AR, Margetts SML and Barrowcliffe TW (1997) International Normalized Ratio determination using calibrated reference plasmas. British Journal of Haematology **98**, 74-78.

6) Campbell PJ (1974) International biological standards and reference preparations. II: Procedures used for the production of biological standards and reference preparations. J Biol Standardisation **2**, 259-267.

7) Kirkwood TBL and Tydeman MS (1984) Design and analysis of accelerated degradation tests for the stability of biological standards II: A flexible computer program for data analysis. J Biol Standardisation **12**, 207-214.

8) Hubbard AR, Margetts SML, Weller LJ, Macnab J and Barrowcliffe TW (1999) An international collaborative study on the INR calibration of freeze-dried reference plasmas. British Journal of Haematology **104**, 455-460

10. ANNEX A - PROTHROMBIN TIMES ESTIMATES OBTAINED IN THE ACCELERATED DEGRADATION STUDIES

Table 10.1 - Prothrombin Time Estimates (seconds) with Manchester Reagent Thromboplastin: BCR 630

Storage time	*Storage temperature (°C)*				
(months)	-20	+4	+20	+37	+45
1	14.4	----	14.4	18.0	27.1
	(14.2 - 14.6)		(14.3 - 14.5)	(17.7 - 18.1)	(26.6 - 27.8)
3	14.3	----	14.8	22.1	----
	(14.1 - 14.4)		(14.6 - 15.1)	(20.9 - 23.2)	
7	14.6	14.6	15.7	31.7	----
	(14.3 - 15.0)	(14.4 - 14.8)	(15.4 - 16.2)	(28.5 - 33.9)	

Table 10.2 - Prothrombin Time Estimates (seconds) with Manchester Reagent Thromboplastin: BCR 631

Storage time	*Storage temperature (°C)*				
(months)	-20	+4	+20	+37	+45
3	33.9	----	36.4	44.6	63.8
	(32.9 - 36.0)		(36.1 - 37.0)	(43.0 - 45.9)	(62.6 - 65.4)
5	34.9	----	39.5	49.5	73.6
	(34.0 - 35.9)		(39.1 - 40.0)	(49.3 - 50.0)	(71.3 - 76.0)
8.5	33.3	34.8	37.7	50.8	----
	(32.8 - 33.9)	(33.1 - 35.6)	(36.7 - 38.8)	(47.1 - 54.6)	

Table 10.3 - Prothrombin Time Estimates (seconds) with Manchester Reagent Thromboplastin: BCR 628

Storage time	*Storage temperature (°C)*				
(months)	-20	+4	+20	+37	+45
2.5	48.5	----	50.3	60.8	74.0
	(47.4 - 49.4)		(50.2 - 51.7)	(60.3 - 61.4)	(73.1 - 77.2)
3	47.9	----	----	60.5	----
	(45.8 - 50.1)			(57.8 - 62.6)	
4.5	49.7	----	53.8	63.1	86.2
	(47.9 - 51.7)		(52.3 - 54.5)	(62.4 - 64.0)	(82.2 - 90.5)
8	47.9	47.6	51.0	67.2	----
	(45.8 - 50.1)	(46.4 - 48.9)	(49.3 - 52.5)	(65.1 - 69.3)	

Prothrombin time estimates are the mean values from at least 6 independent determinations. The range of estimates are given in the brackets.

Table 10.4 - Prothrombin Time Estimates (seconds) with Baxter Innovin Thromboplastin: BCR-630

Storage time	*Storage temperature (^oC)*				
(months)	-20	+4	+20	+37	+45
3	10.2	----	----	14.7	----
	(10.0 - 10.3)			(14.1 - 15.6)	
4	10.1	----	10.8	15.5	----
	(10.0 - 10.4)		(10.6 - 11.1)	(14.8 - 16.0)	
7	10.2	10.4	11.1	19.0	----
	(10.0 - 10.3)	(10.2 - 10.5)	(10.9 - 11.3)	(18.5 - 20.1)	

Table 10.5 - - Prothrombin Time Estimates (seconds) with Baxter Innovin Thromboplastin: BCR-631

Storage time	*Storage temperature (^oC)*				
(months)	-20	+4	+20	+37	+45
3	26.9	----	----	32.5	----
	(26.4 - 27.3)			(31.9 - 33.0)	
6	26.8	----	28.3	33.7	46.5
	(26.6 - 27.0)		(28.0 - 28.7)	(33.3 - 34.0)	(44.4 - 48.0)
8.5	26.9	27.3	28.6	36.3	----
	(26.4 - 27.3)	(26.8 - 27.7)	(29.3 - 28.1)	(35.7 - 36.9)	

Table 10.6 - Prothrombin Time Estimates (seconds) with Baxter Innovin Thromboplastin: BCR-628

Storage time	*Storage temperature (^oC)*				
(months)	-20	+4	+20	+37	+45
3	45.0	----	----	51.7	----
	(44.1 - 46.0)			(50.3 - 52.4)	
5.5	45.7	----	47.4	54.6	70.5
	(45.6 - 46.1)		(47.1 - 47.5)	(54.4 - 54.9)	(69.6 - 72.0)
8	45.0	44.9	46.9	56.3	----
	(44.1 - 46.0)	(44.0 - 46.4)	(46.6 - 47.6)	(55.0 - 58.5)	

Prothrombin time estimates are the mean values from at least 6 independent determinations. The range of estimates are given in the brackets.

European Commission

EUR 21060 – DG Joint Research Centre, Institute for Reference Materials and Measurements –
The certification of reference plasmas for the prothrombin time, BCR-628 (Abnormal Plasma 2), BCR-630 (Normal Plasma), BCR-631 (Abnormal Plasma 1)
T.W. Barrowcliffe, A.R. Hubbard, S. Margetts, L.J. Weller, J. MacNab, D. Bennink, B.M. Gawlik, C.L. Klein, A. Lamberty
Luxembourg: Office for Official Publications of the European Communities
2004 –41 pp. –21.0 x 29.7 cm
Scientific and Technical Research series
ISBN 92-894-5172-6

Abstract

The aim of the present project was to provide an alternative method of estimating INR (International Normalised Ratio) which will lead to increased inter-laboratory agreement and also overcome the need for time-consuming local calibration exercises which are currently required to compensate for differences in instrumentation and reagents. It is proposed to use three freeze-dried pooled plasmas (one normal plasma pool and two abnormal plasma pools with increased INR values) each with an assigned INR value to construct a calibration curve by plotting local prothrombin time (PT) vs. assigned INR value. The INR of test plasmas can then be interpolated from local PT estimates.

Three definitive preparations consisting of a pooled normal plasma and two pools of patient plasma (1 medium and 1 high INR range) were prepared for INR calibration in a collaborative study involving 20 laboratories. Estimates of homogeneity (by weight and PT estimation) and stability indicated that the preparations were suitable for use as reference preparations. INR values were estimated using three International Reference Thromboplastin preparations (BCT/253 - human, RBT/90 - rabbit, OBT/79 - bovine). Inter-laboratory variability of INR estimates was lowest with the normal plasma (maximum GCV 4.32%) and highest with the high INR patient plasma (maximum GCV 10.99%). The low variability was the main reason for significant differences between some mean INR estimates with different thromboplastin reagents. The largest difference was seen with abnormal plasma-2 between the INR estimate with OBT/79 (3.20) and the estimates with RBT/90 (3.93) and BCT/253 (3.75) and this invalidated the assignment of a mean INR value calculated from the results obtained with all three thromboplastin reagents. The assigned INR values are the geometric means of the INR estimates obtained by the individual laboratories using BCT/253 and RBT/90. These assigned values are intended to be used with all thromboplastin reagents of human or rabbit origin.

The mission of IRMM is to promote a common European measurement system in support of EU policies, especially health and consumer protection, environment, agriculture, internal market and industrial standards.

European Commission
Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements

Contact information
European Commission
Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements
Retieseweg 111
B-2440 Geel • Belgium

Tel.: +32 (0)14 571211
Fax: +32 (0)14 590406

http://www.irmm.jrc.be
http://www.jrc.cec.eu.int

EUR Report 21060
Luxembourg: Office for Official Publications of the European Communities

ISBN 92-894-5172-6

Printed in Belgium

The mission of the Joint Research Centre is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of European Union policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Community. Close to the policy-making process, it serves the common interest of the Member States, while being independent of commercial and national interests.

EUROPEAN COMMISSION
DG JRC
Joint Research Centre

15 LA-NA-21060-EN-C

ISBN 92-894-5172-6

9 789289 451727

Publications Office
Publications.eu.int